ADOPTIVE T CELL THERAPY

IN SOLID TUMORS

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ABSTRACT:

Cancer therapy proceeds by means of immune surveillance of T cells as their receptors scan human leukocyte antigen presenting molecules. Artificially designed T cell receptors can be used to target tumor specific antigens. Preclinical advantages are discussed and clinical achievements displayed, involving cost-effective research and more specific targeting to improve efficacy. New insights in stem cell biology influence this field of research and I will discuss the perspectives of immunology, oncology and stem cell biology combined.

PROLOGUE

In this review on adoptive T cell therapy very different fields in the science of biology research and its translation into medical applications meet. The ongoing research in cancer treatment has lead to broadening approaches such as biologicals and has now come to humanly owned defenses by the means of the immune system. This system normally performs excellent surveillance in the human body as it eradicates every particle that is unknown and thereby potentially harmful to ensure optimal physical functioning of the human body. In the case of cancer this immensely complicated system has somehow failed to recognize the tumor cells which results into unrestrained proliferation of damaged cells.

The implications of cancer on morbidity and mortality worldwide are far-reaching and many individuals have encountered the effects of it not far off. This makes the research a fortiori weighty which is to me a major reason to be interested in the development of new treatments.

Furthermore, the immune system is an immensely complex entirety in which every single cell and molecule work together in concert. I think this is fascinating as it displays an intriguing greatness.

A third reason for me to make a study of the development of adoptive T cell therapy is the upcoming use of T cells with stem cell like properties. One stem cell has the potential of repopulating an entire human body with a new, functional hemopoietic system, which I think is fascinating.

The interface of these fields, oncology, immunology and stem cell biology, has inspired me to make a study of the upcoming treatment with adoptive T cells. The field is quickly developing as many researchers are working to make this promising T cells clinically available for every cancer patient.

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Adoptive T-cell therapy – an overview

Cancer is a major cause of death worldwide accounting for 7.6 million deaths per year. An increase in incidence to 13.1 million deaths in 2030 is expected. Incidence varies along country, sex, affected organs and is associated with high body mass index, low fruit and vegetable intake, lack of physical exercise, tobacco use and alcohol use. The highest mortality rates are seen in low and medium developed countries (*J. Ferlay, H. Shin, 2008*). The mortality rate is globally seen as a measurement for treatment effectiveness as it reflects the impact on a population, compared to the diagnoses made in the same year (*M.P. Coleman, J. Estève, 1993*). Since an increase is expected, the quest for improved cancer treatment continues.

Current therapies

Treatment of cancer nowadays often includes radiation therapy, chemotherapy and surgery. Radiation can be aimed specific at the tumor to induce DNA damage, making the tumor genetically unstable. This will trigger apoptosis in many tumor cells. Side effects are collateral damage caused by the radiation, since adjacent tissue also gets affected. Chemotherapy aims for quickly dividing cells like cancerous cells by stopping mitosis. This causes major side effects in many other cells, like bone marrow, intestine lumen and hair follicles that similarly rely on high proliferation (*Y. Chen, P. Jungsuwadee, 2007 , S. A. Lorimore and E. G. Wright, 2003*). Novel therapies with improved anti-cancer activity and a move favorable toxicity profile are highly wanted. For chemotherapy, the aim is to have a more specific target, to affect only cells expressing tumor-specific proteins instead of all quickly dividing cells. This type of medication is less harmful as it does not affect healthy tissue as classical therapy does *(D. Canals, Y.A. Hannun, 2013).* Research focuses on new proteins and current targets are specified to optimize targeted therapy.

Apart from these "classical" treatments, immunotherapy particularly for chronic myeloid leukemia and melanoma has proven highly effective and these findings are currently being extended to other forms of cancer. Below, I discuss the background of the immune system, its interaction with cancer and exploitation for therapy of solid tumors.

Tumor antigens and the immune system

A human body contains millions of T cells but per antigen only a few T cells circulate. It requires a strongly regulated mechanism to achieve a sufficient immune response. Also, a too high response would be toxic to the body, so fine-tuning of this response is essential. The mediators of this mechanism are human leukocyte antigens (HLA) present on virtually every nucleated cell and the T cell receptor (TCR) on the membrane of the T cell. This interaction leads to a cascade of events

resulting in deletion of cells expressing the antigen (from *Cellular and Molecular Immunology, 7th edition*).

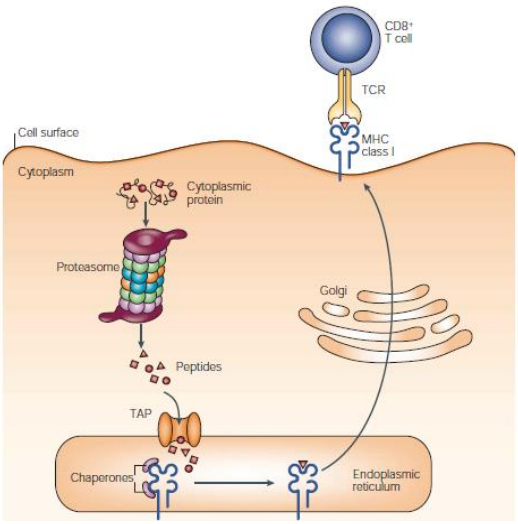
The function of HLA's

First of all, HLA's are designed as unique molecules for every human by combining different parts of the HLA genes. These genes are the most polymorphic genes present in the genome. HLA molecules occur in two classes, I and II which are structurally different but homologous. HLA class I is present on virtually every nucleated cell. Class II HLA molecules are only expressed on dendrite cells, B lymphocytes, macrophages, and a few other cell types involved in the immune system. Class I is recognized by $CD8^+$ T cells whereas class II is recognized by $CD4^+$ T cells. Both CD4 and CD8 are co stimulators of the T cell receptor (TCR). The fold of HLA molecules is very specific as it binds proteins from within the cytosol before presenting them on the membrane. The interactions performed by HLA molecules are crucial in an individual's health, not only in immune regulation *(E.J. Adams, A.M. Luoma 2013).* This article will mainly focus on $CD8^+$ T cells.

The structure of HLAs

Every HLA molecule contains an extracellular peptide-binding groove, followed by immunoglobulin (Ig)-like domains, transmembrane domains to anchor the molecule and cytoplasmic domains for cell signaling (fig. 1). The peptide-binding groove contains the polymorphic amino acid residues of HLA molecules. The non-polymorphic Ig-like domains of HLA molecules are recognized by the coreceptors expressed on T cells. A class I HLA molecule, when fully assembled, consists of a heterotrimer consisting of an α chain, β_2 microglobulin, and a bound antigenic peptide. All three components need to be present to ensure stable expression on a cell's surface.

Fig. 1. Intracellular HLA pathway. *The class I pathway processes protein antigens in the cytosol. Proteasomes cut proteins and process them to the endoplasmatic reticulum where they non-covalently bind to the binding groove of class I HLA molecules. These do not discriminate between foreign proteins and proteins derived from the individual self. The structural conformation of proteins is lost as T cells only recognize the amino acid sequence. Vesicles containing HLA molecules are processed to the plasma membrane to be recognized by $CD8^+$ T –cell (J. W. Yewdell, E. Reits et al. 2003).*



HLA and peptides. HLA molecules show a broad specificity for peptide binding, in contrast to the fine specificity of antigen recognition of the antigen receptor of T lymphocytes. Each class I or class II molecule can bind many different peptides in their single cleft for peptide binding. The peptides that bind to HLA molecules share structural features that promote this interaction. The association of antigenic peptides and HLA molecules is a saturable interaction with a very slow off-rate. This will lengthen the display duration and thereby maximize the chance that a specific T cell will find the peptide and initiate a response (*J. G. Bodmer, 1978)*.

For presentation of antigens to T cells, dendritic cells, also known as antigen-presenting cells (APC) are required. Their name is derived from the Greek word for branch as these cells are seen to 'branch' towards an antigen to capture and present it. APC's display peptide-HLA class II complexes for recognition by T cells and also provide additional stimuli to the T cells such as CD28, CD80 and CD86, that are required for a full response. The dendritic cells migrate towards the draining lymph nodes for a chance to meet their specific T cells. Once a T cell is activated by an APC, it will be stimulated to proliferate and differentiate to achieve a full immune response *(J. E. Smith-Garvin, G.A. Koretzky et al, 2009).*

The T cell receptor

The receptor for HLA molecules on the plasma membrane of a T cell is called the TCR. It is a heterodimer, most frequently consistent of an α and a β chain. The chains are composed of a variable and a constant part. The constant part consists of the part binding to the membrane and a small domain protruding into the cytosol. The variable region is the part that can recognize HLA molecules loaded with a specific peptide. In this region, there are three hyper variable regions, called the complementarity determining regions (CDRs). Each region has its own role in antigen recognition although CDR3 is thought to play the major role in this process.

Two signal model

Upon recognition of HLA the T cell will be activated to start the immune response to kill cells presenting the antigen. However, HLA stimulation needs triggering of co stimulators as well for proper stimulation. CD28 is a molecule expressed on T cells to enhance the effect of HLA signaling. It binds to B7.1 and/or B7.2 (CD80 and CD86, respectively) present on the antigen presenting cell (APC) (*F.A. Harding, J. P. Allison et al, 1993*). It stimulates the expression of IL-2 which leads to maturation of the naïve T cells. These are not the only molecules involved in co stimulation. In fact, there are two super families of immunoglobulins (B7) and tumor necrosis factor (TNF) important. These signaling molecules are homologous but structurally quite diverse. The complexity of the stimulating and inhibiting properties is incredibly complex. A more elaborate view on the B7 family is

given by *P. Greaves. J.G. Gribben, 2013* and the role of TNF is reviewed by *W.M. Chu, 2013.* In the figure below, the interaction between dendritic cell and naïve T cells is shown.

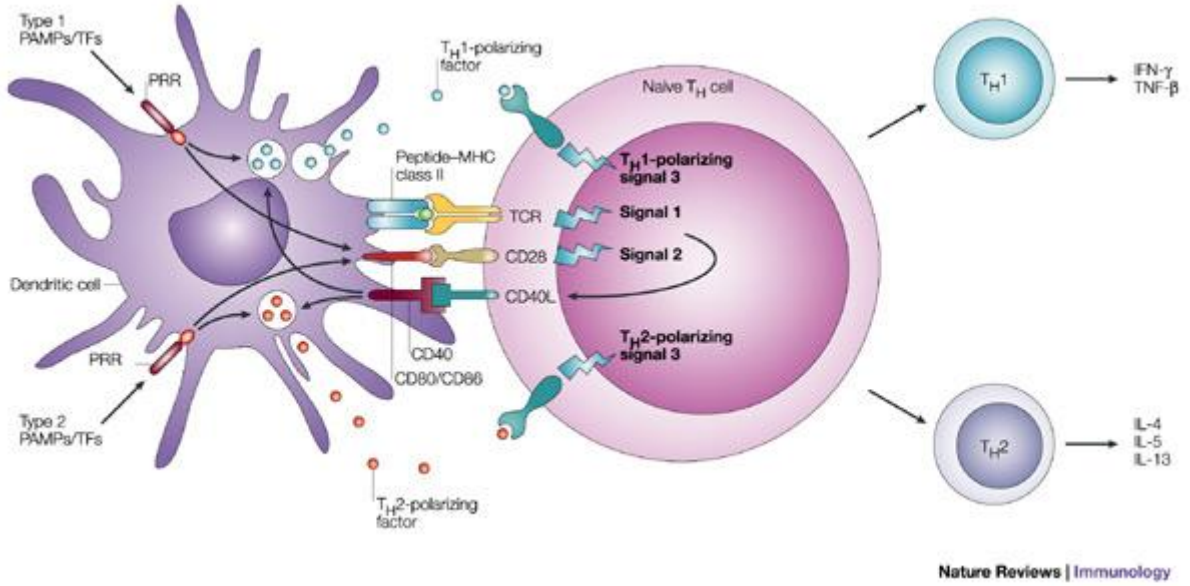


Fig. 2. An overview of a dendritic cell and T_H interaction and the 2/3 signal model. *The TCR mediates the antigen specific signal, signal 1, after (TCR) being triggered by MHC class-II-associated peptides. These are processed from pathogens after internalization through specialized pattern recognition receptors (PRRs). Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 (B7.1 and B7.2) that are expressed by dendritic cells (DCs) after ligation of PRRs, such as Toll-like receptors (TLRs) that are specialized to sense infection through recognition of pathogen-associated molecular patterns (PAMPs) or inflammatory tissue factors (TFs). Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as interleukin-12 (IL-12) and CC-chemokine ligand 2 (CCL2), that promote the development of T_{H1} or T_{H2} cells, respectively. The nature of signal 3 depends on the activation of particular PRRs by PAMPs or TFs. It The profile of T-cell-polarizing factors is primed by recognition of PAMPs, optimal expression of this profile often requires feedback stimulation by CD40 ligand (CD40L) expressed by T cells after activation by signals 1 and 2. IFN-β, interferon-γ; TNF-β, tumor-necrosis factor-beta. (M.L. Kapsenberg, 2003).*

Spontaneous tumor regression

Early evidence for a possible role of the immune system in the control of cancer came from the observation of spontaneous tumor regression in patients. In very rare cases (1 out of 60.000 – 100.000) spontaneous tumor regression of a proven malignant tumor is observed without the intervention of treatment in any form (*T. C. Everson. 1964*). Various reasons for this observation have been given, varying from psychological to immunological origin. The latter is thought to be mediated by tumor-

antigen specific T cells as T-lymphocytes are observed to invade solid tumors (TILs). This observation has led to detailed research towards using these cells for adoptive T cell therapy (ACT).

The immune system and cancer

When a cell has undergone specific DNA mutations it can become a cancer cell. This is visible by numerous hallmarks, for example aberrant expression of normal proteins in HLA molecules of tumor peptides. A classification in this tumor antigens is based on this expression pattern. Peptides only expressed on tumor cells are called *tumor-specific antigens*. Tumor antigens that are also expressed on normal cells, but overexpressed on cancer cells are called *tumor-associated antigens*. Altered proteins will be processed in the HLA class I presenting pathway and become visible for APCs and $CD8^+$ T cells. However, many tumors tend to be only weakly immunogenic. This varies with the type of tumor, and also the tumor can outgrow the immune system's ability to cope with the immunogenicity of the tumor. Furthermore, many cancer cells avoid this system by stopping the immune process or by not expressing HLA molecules at all. Still, observations have shown that tumor sites are surrounded by cells from the immune system (fig. 3).

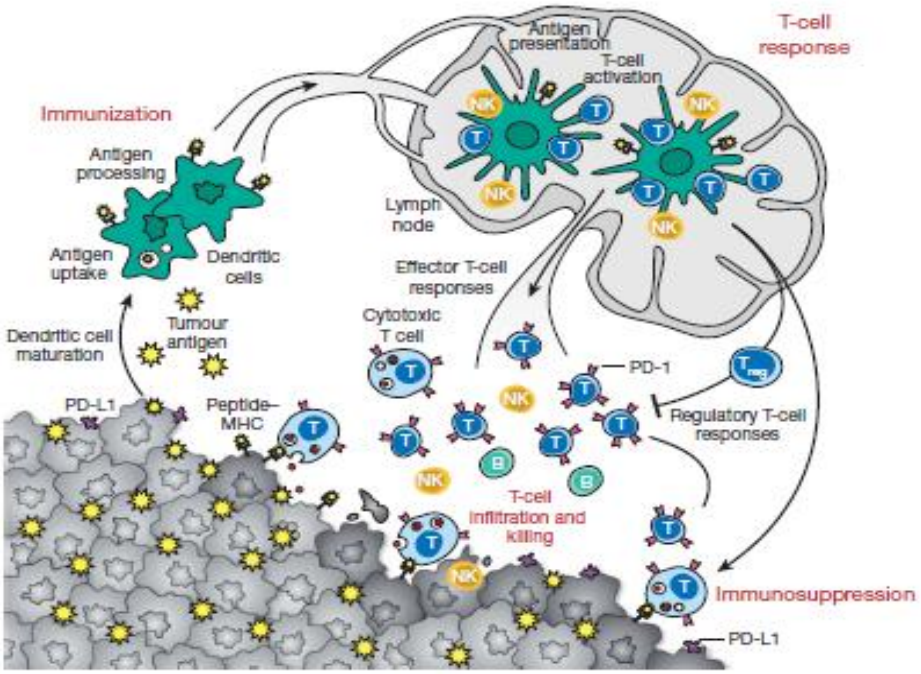
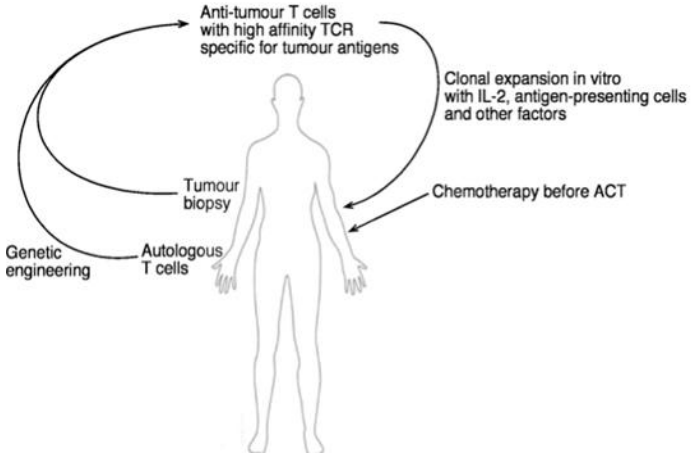


Fig. 3. *Dendritic cells take up tumor antigens and migrate to the lymph node, a "meeting point" for many immune cells. There it activates T cells specific for that antigen to proliferate and attack all cells expressing that specific antigen. Many different sub-types of T-cells are involved (I. Mellman, G. Coukos, 2011).*

Adoptive T cell therapy

Fig. 4. *Schematic overview of adoptive T cell therapy*



These immune cells around tumor sites can be extracted and kept *ex vivo*. Using sorting techniques, tumor specific $CD8^+$ T cells can be extracted and identified from this tumor infiltrate or from the blood. They are *ex vivo* activated to expand the population. After growth, these cells can be transferred back into the patient to obtain an increased anti-tumor response. This therapy is called adoptive T cell therapy (ACT) (fig. 4). Rosenberg and colleagues have established a long-term effect of tumor regression after treating cancer patients using this method, by demonstrating durable regression of melanoma tumors *(S.A. Rosenburg, M.E. Dudley, 2009).* These results are promising for patients, although many limitations remain to be overcome.

This review will focus on the achievements made in ACT preclinically and will the discuss the clinical trials preformed in this field. Finally, I will discuss my personal view on the future perspectives in this field of research.

Preclinical results

T cells that leave the blood stream and invade a tumor are often correlated with a better prognosis. As said before, these cells can be taken out of the patient and cultured *ex vivo*. At first, this was purely performed for studying immune effector cells. This experiment demonstrated differences between tumor infiltrating lymphocytes and peripheral lymphocytes. Especially the functional capacities are elevated in tumor infiltrating lymphocytes, although it was clear that the tumor microenvironment is inhibitory for many of the lymphocyte functions as they were visibly restricted from acting (*E.C. Holmes, 1985)*. Later on, the studies were aimed at using the tumor infiltrating lymphocytes as a therapy for cancer treatment. As this technique was shown to be effective, more approaches were designed to make optimal use of the possibilities.

Tumor infiltrating lymphocytes

At the start of modern cancer research, it was thought that a tumor was a homologous entity without any intern variation as it originated from one single mutated cell. Nowadays it is known that a tumor consists of many differentiated cell types and interacts with its environment to maintain and nurture itself. It is surrounded by cells from the immune system which are attracted by the distorted malignant growth. However, they are not functional as they are deregulated by the tumor. These are immune cells that can be isolated and can have anti-tumor effects when they are present in higher quantities *(E. Klein, S. Becker, 1976).*

TCR-T cell technique

Modification of T-cells can be achieved using two different approaches. The first discussed here is the modification of the T-cell receptor that recognizes HLA. Genes that encode for the α and β chain of the TCR can be isolated from rare patients that show spontaneous tumor regression. By means of viral transduction these genes are introduced into T-cells and large numbers of antigen-specific T-cells, called TCR-T cells can be generated. These cells not only respond to tumor cells but also secrete Th-1 helper cell cytokines, they proliferate and can directly kill a target cell. This shows that the cells actually are functional (*R.A. Morgan, M.E. Dudley et al, 2003*).

Improvements of TCR-T cell technique

Improvements in design. Affinity of T cell receptors obtained by expression of artificial genes is often lower than conventional T cells. To make TCR-T cell therapy more efficient the receptor is adjusted to obtain an affinity similar to conventional T cells. Substitution of certain amino acids in the CDRs of TCRs leads to enhanced recognition of antigens. Especially substitutions in CDR3 are effective (*P.F. Robbins, Y.F. Li, 2008*). Introduction of cysteines to form interchain disulfide bonds

can prevent the mispairing of the introduced TCR chains and the endogenous chains *(P.F. Robbins, S.A. Rosenberg et al. 2008).* Improvement in design also leads to an increase in expression on the cell surface of TCR *(M.A. de Witte, A. Jorritsma, 2008).* These effects are similar to an increase in affinity for the antigen described above. Exogenous and endogenous TCR chains compete for expression on the cell surface. The success of this competition varies with the type of antigen *(L. Chen, D.B. Flies, 2013).*

Improvement in gene transfection. The genes encoding for specific TCRs can be transfected in different ways. Commonly, viral or retro-viral approaches are used *ex vivo* after which the T cells are placed back into the patient. These viruses contain vectors encoding the desired TCR *(J.D. Suerth, A. Schambach et al. 2013).* Research is focused on making this technique more effective to guarantee an elevated product consistency and on finding less elaborate alternatives.

Improvement of environment. Administration of exogenous cytokines like IL-2 increases persistence of transferred T cells. It makes the cells proliferate and differentiate towards effector cells which increases their efficacy. Tumors are commonly surrounded by immune cells, but these are often not properly functional. This is probably due to cytokines secreted by the tumor to inhibit an immune response *(J.A. Westwood, M.H. Kershaw, 2010)*.

T-cell therapy modified using CARs genes

This approach is different from the one described above in the way that tumor associated antigens (TAA) are targeted. Here, chimeric antigen receptors (CARs) that recognize TAA through single-chain variable fragments (scFvs) that are isolated from antigen-specific monoclonal antibodies (mAbs) are used. This overcomes a major obstacle in the TCR-T approach, where the recognition is restricted to a single HLA type. CARs are constructed by attaching a scFv region to T cell intracellular signaling chains; transducing the resultant molecule into T cells enables these effector cells to recognize targets using antigen recognition of the antibody. Current CARs are grouped into three generations, with increasing costimulatory activity. The first generation lacked intracellular signaling and had little clinical activity, to be used as a test model. The next generation used the 2 signaling model from the T cell as a costimulatory signal, including molecules such as CD28. The third generation has improved signaling capacities compared to the previous generation (*H. Shi, L. Liu, 2013)*.

Improvements in CARs

Spacer/hinge regions. Not only the costimulatory signaling is crucial in CAR improvement, also the spacer/hinge region plays an important role. It is found between the antigen-binding and TM domains and mediates CAR flexibility and thereby facilitates the positioning of the binding domain during scFv-antigen interactions. This observation took place when comparing different spacer/hinge regions in similar CARs *(D. Moritz, B Groner 1995)*.

In vivo survival and expansion. Compared to normal T cells, CAR-T cells have reduced proliferation and survival. It is important to increase this to ensure a larger therapeutic efficacy. When T-cells are less differentiated, like naïve or central memory T cells, they have a greater proliferative capacity in vivo *(C.H. June, 2007)*. T-cells can be enriched before CAR introduction, using cell surface molecules associated with less differentiated states, such as CD62-ligand, to maintain these less differentiated properties.

The chemokine system. Chemokines are small chemoattractive proteins commonly found in soluble form. Attracting T and B cells occurs via a gradient of chemokines to tissue where this is required, called homing. Often a broad range of immune cells is observed around the tumor of which only a few are functional. This is due to the immune suppressive characteristics of tumors *(I. Marigo, L. Dolcetti, 2008)*. Migration of the adoptive T-cells to the cancer site is crucial for the efficacy of therapy. Therefore, new approaches could include co-expression of chemokines on the surface of CARs. The feasibility has been shown by *M.H. Kershaw, G. Wang et al in 2002*. The challenge is to target a specific chemokine secreted by the tumor and to design the appropriate receptor on the T cell.

Universal CAR+ T cells. Graft versus host disease (GHVD) is a major problem in adoptive T cell therapy. It means that the T cells attack not only the tumor cells but also healthy tissue which leads to severe health problems. Therefore, for every patient it is necessary to design a T cell specific for their type of tumor. Recently however, universal allogenic TAA-specific T cells are designed that might be administered to multiple recipients. Expression of CD19 is upregulated and the endogenous αβ TCR receptor expression was suppressed, without compromising CAR-specific effector functions. By eliminating endogenous αβ TCR the chance on a graft-versus-host response can be decreased. This could imply that such T cells can be designed in one donor and administered in multiple patients *(H. Torikai, A. Reik, 2012)*. More targets than solely CD19 could be designed to make this approach broader applicable. It is under investigation how to avoid depletion by natural killer cells that attack non-autologous HLA molecules expressed on the surface.

Immunogenecity. A part of the CARs is a chimere, because most scFvs are derived from mouse monoclonal antibodies. This causes the human body to produce human anti-mouse antibodies which has side effects varying from rashes to renal dysfunctioning due to an allergic response. The antibodies can be humanized but this still causes some HAMA's to form. A way to overcome this is to design an antibody binding site using endogenous protein ligands or receptors. Treatment of mice with these antibodies significantly increases life span compared to controls treated with conventional IgG treatment. In this way no immunogenic response in expected *(Q. Ye, Z. Wang et al, 2011).* This could be an option for the design of new CAR models and could form a new approach for treatment of GHVD.

Improvements in safety in TCR T-cells and CARs

The development of TCR T cells and CARs mainly focuses on safety. Toxicity is an important issue to overcome in the clinic and often cross-reactions between normal cells expression the isolated antigen are observed. Therefore, choosing the proper affinity is crucial. To avoid using the chemokine system for attracting the correct T cells is to administer the T cells operatively on the tumor site. This decreases 'on-target, off-tumor' immune responses and increases the efficacy (*H. Shi, L. Liu, 2013).*

A major disadvantage of adoptive T cell therapy in general is the possibility of reaction of these newly introduced T cells to react against non-tumor tissue. This causes severe damage in the patient, since the T cells are in extremely high amount present whereas normally this would be regulated by the immune system. This excess quantity causes disturbances in many organs and can be accompanied by high morbidity and mortality. Therefore it would be a major advantage to have a switch on the functionality of these T cells. This has been designed in the form of suicide genes. When a reaction in a patient is too strong, a trigger for these genes can be administered to directly stop the immune response by killing all the injected T cells instantly. This could prevent a cytokine storm and death. An example of this gene is the one encoding for caspase 9. A too strong reaction has been reported to be stoppable in the time span of only 30 min *(V. Marin, E. Cribioli et al. 2012).*

In normal T cell reactions there are checkpoints in proliferation to guarantee an optimal immune response. These can be included in artificially designed T cells as well by the means of subsets with different costimulatory signaling domains. Often, DNA transfers have been used to adjust the T cell receptor or introduce a chimere receptor. This will increase the specificity and thereby the efficacy of adoptive T cell therapy.

Recently, safety research has lead to the use of T cells electroporated with mRNA's. This appears to be safer as the toxicity is expected to abate rapidly. As this method has not passed the clinical trials yet, it dose-escalated schemes should be designed to guarantee an optimal response from every patient. This may prevent severe toxicity such as cytokine storms *(D.A. Mitchell, I. Karikari et al. 2008).*

T cell subsets

As shown in fig. 2, not only $CD8^+$ T cells are involved in this therapy. When performing ACT, it is important to administer the right cells to have the most effective results. Beyond Ag specificity, T cells are heterogeneous with respect to a spectrum of other parameters such as proliferation, cytokine secretion and gene expression profiles. Each of these parameters may independently influence a T cell's ability to mediate cancer regression after ACT. There are two commonly used methods to select specific T cells which will be described below.

FACS. A fluorescence activated cell sorter can be used to investigate the prevalence of specific membrane proteins by the means of lasers screening for fluorescent tags. Investigated cells can be kept alive during the analysis guaranteeing thereby a minimal loss of specific T-cells. Not only Ag-specific

T cells can be selected but also the developmental stages can be determined, dependent on the fluorescent label of the FACS.

MACS. A magnetic activated cell sorter can distinguish cells based on the expression of their surface antigens (CD molecules). Magnetic beads are coated with antibodies and thereby are highly selective, after which the sample is placed in a strong magnetic field to sort the cells. This field is less developed but can be as specific is FACS by using similar antibodies.

Mutanoom

Identification of mutated proteins in tumor cells is a laborious job considering the fact that complete DNA libraries have to be created. Recently, S.A. Rosenberg et al. have designed a set-up by sequencing solely the exome and not the complete DNA. In a clinical trial with TIL, 70% of the patients showed substantial regression in metastatic lesions and 40% of the patients in another trial showed complete regression after the administration of TIL. The team has investigated the potential correlation between recognition of specific mutated genes and specificity of T cells. Comparing whole exome sequences of non-affected tissue and tumor cells, non-synonymous somatic mutations have been identified. Scanning the MHC-bound peptides gave insight in which proteins are most often mutated. These are synthesized and tested for efficacy by monitoring interferon γ release by lymphocytes *in vitro*. The specific proteins are found to be mutated in a way that enhances their MHC-binding affinity which makes them a more expressed protein compared to WT. Evaluating samples on these specific proteins before and after treatment indicates a correlation between these mutated proteins and TIL efficacy.

This method provides a simple and rapid approach for TIL target identification and gives insight in the relation between the clinical efficacy and recognition of epitopes. These can also be used for sensitization and expansion of TIL *in vitro*. Eventually, it might also become applicable in multiple cancer types *(P.F. Robbins, S.A. Rosenberg, 2013).*

Clinical results of adoptive T-cell therapy

Tumor infiltrating lymphocytes

The first clinical results of tumor infiltrating lymphocytes (TIL) were obtained by Rosenberg et al. in a clinical study with thirteen patients with progressive disease refractory to standard therapy. The T-cells used for treatment were isolated from the tumor and rapidly expanded in vitro. Six patients out of thirteen had objective clinical responses after transfection and four others displayed mixed responses. Objective tumor regression was observed which displays the efficacy of this treatment. This was one of the first experiments around adoptive T cell therapy and has been fine-tuned over time as described below in clinical trials with adaptations compared to the method described in this trial .

Combination of TIL treatment with IL-2

The first attempts to adjust T cell therapy was performed by means of administrating IL-2 when T cells were proliferating ex vivo. This molecule will stimulate proliferative and differentiation properties of T cells. In a clinical trial with ninety-three patients with metastactic melanoma's, autologous adoptive T cells were administered after lymphodepletion (chemotherapy or radiaton therapy) . Overall survival of patients with metastatic melanoma's is low and durable regression after TIL therapy is rare. Twenty of these patients achieved a complete tumor regression of which nineteen still had this regression after three years. The overall survival rate after three and five years is 36% and 29% respectively but for the patients with complete response 100% and 93% respectively. The treatment has similar effects irrespectively to prior treatment. A negative effect however is the effect IL-2 has on differentiating as it drives this process forward. Therefore the capacity of ex vivo T cells decreases *(S.A. Rosenberg, M.E. Dudley, 2011)*. More about progenitor cells and alternatives for IL-2 in TIL therapy will be discussed in the last chapter.

TIL and functional impairment by PD-1 – inhibition

Programmed cell death 1 (PD-1) is a cell surface protein that activates apoptosis upon ligand binding. This decreases proliferation in T cells when expressed. The research group of S.L. Topalian (jan. 2013) has investigated the effects of tumor infiltrating lymphocytes when combined with administration of PD-1 blocking antibodies. This is a phase I first in human trial on patients with treatment-refractory solid tumors. Three patients were followed after treatment for three years. A complete response and a partial response was observed. The recurrent disease after partial response was successfully treated with reintroduction of PD-1 inhibitors. The results of this research suggest a reset of the immune interaction between tumor and host as the PD-1 therapy appears to restore the function of T cells with long term memory, which causes an ongoing anti-tumor effect even in the absence of treatment. A recurrence of tumors may reflect a disturbance in this equilibrium, where new

administration will lead to a new balance and therefore tumor regression *(E.J. Lipson, S.L. Topalian, 2013).*

In-patient stimulation of T-cells

For above mentioned treatments, tumor cells are surgically acquired, which is a major source of stress in cancer patients. A strategy to overcome operating these severely weakened patients is to stimulate T cells with a whole tumor vaccine in vivo. Immune surveilling cells can pick up tumor particles and will present specific molecules to T-cells. This approach has thus far better clinical responses than highly specific tumor vaccines in various types of cancer. An important part of this therapy is to deplete the host's immune system with cyclophophamide. This deregulates T-regs which are known to promote immune dysfunction in tumors. Furthermore, it has some anti-angiogenesis effects. Complete understanding of this process is not known yet, The research group of *G. Coukos (jan 2013)* has performed a study where whole tumor cell-based vaccination, using lysate-pulsed autologous dendritic cells in combination with bevacizumab and metronomic yclophosphamide, followed by autologous adoptive T-cell therapy using vaccine-pried CD3/CD28-costimulated whole peripheral blood lymphocytes, are used to treat patients with advanced cancer stages. 4 out of 6 patients achieved clinical benefits with this combination. The patients participating in this trial have been treated with bevacizumab and cyclophosphamide before and have failed to respond to this treatment. Therefore the therapeutic success cannot be attributed solely to anti-angiogenic therapy of cyclosphosphamide in this setting. The important question still remains whether the adoptive transfer of vaccine-primed co-stimulated T cells is an effective way to boost the efficacy of cancer vaccines.

CAR therapy

The need for specific targets for T cells is indicated by a study of S.A. Grupp and C.H. June (april 2013). Here, CAR T cells with a specificity for CD19 have shown promising results in the treatment of chronic lymphocytic leukemia. Two children with relapsed and refractory pre-B-cell acute lymphoid leukemia received infusions of T cells transduced with anti-CD19 antibody and a T-cell singaling molecule (CTL019 chimeric antigen receptor T cells). The T cells expanded to a level that was more than 1000 times as high as the initial engraftment level. The cells were observed in bone marrow and in cerebrospinal fluid. The patients reacted with a cytokine-release syndrome and a macrophage activation syndrome. This was expected, since a severe impairment of this strategy is the toxicity. It was treated and overcome without effect for the injected CAR T cells. Complete remission was observed in both patients and is ongoing in one patient 11 months after treatment where the other had a relapse with cells that no longer express CD19 cells. Therefore, a broader range of targets of CAR T cells is needed.

Preclinical trials are performed using VGEF-1a as a target under stimulation of IL-15. The results are positive and thereby providing a rationale for clinical application (W. Wang, Y.Q. Wei, 2013).

As described above, the therapy of adoptive T cell transfer is very young and many aspects and details are yet unknown. Clinical trials are hard to perform as many patients are required to acquire statistically significant results, and participating in such a trial is mentally very challenging.

Table 1. Overview of clinical studies

Malignancy	Response and size of trial	Reference
Colorectal carcinoma	10 patients. No responses, T cells persisted < 10 weeks. Toxicity in 2 patients	R.S. Warren, G.A. Fisher et al., 1998
Colorectal and breast cancer	7 patients. Decrease in serum antigen	Q. Ma, R.M. Gonzalo-Daganzo et al., 2002
Ovarian cancer	14 patients, no response. Tumor localization in one patient	M.H. Kershaw, J.A. Westwood et al., 2006
Neuroblastoma	8 patients. 1 complete response, 3 temporary regression or tumor necrosis. T cell persisted > 6 weeks	M.A. Pule, B. Savoldo et. al., 2008.
Metastatic melanoma	17 patients. 2 objective regression, T cells persisted > 2 months	R.A. Morgan, M.E. Dudley et al., 2006.
Metastatic melanoma	20 patients. 6 objective cancer regression. Allergic response	L.A. Johnson, R.A. Morgan et al., 2009
Metastatic melanoma	16 patients, 3 objective cancer regression	L.A. Johnson, R.A. Morgan et al., 2009
Renal cell melanoma	No response, liver toxicity	C.H. Lamers, S. Sleijfer et al., 2006
Neuroblastoma	6 patients, stable and partial response	J.R. Park, D.L. Digiusto et al., 2007

Proceeding in adoptive T cell therapy

Current methods refined

In chapter 2 the preclinical results of T adoptive T cell therapy are displayed. These refinements of therapy will improve the efficacy and decrease the costs of adoptive T cell therapy which in summary will make the therapy more applicable in clinics.

Safety

The aim of research in the field of adoptive T cell therapy is often safety-related, in both the case of CAR therapy or TCR-T cell therapy. Recently, this has lead to the use of T cells electroporated with mRNA's. This appears to be safer as the toxicity is expected to abate rapidly. This method is not applicable yet as it has not passed clinical trials. In the future, it could be a standardized method to program T cells instead of gene transfer by viral vectors. This may prevent severe toxicity such as cytokine storms *(D.A. Mitchell, I. Karikari et al. 2008)*.

Chimere Antigen Receptor therapy

A recent advance in CAR therapy is the development of universal CAR positive T cells. The major advantage is the avoidance of graft-versus-host disease, as mentioned before. Improvements in this field would make this approach broader applicable as the costs drastically decrease when one type of cell could be administered in multiple patients. Often, these cells are rejected as the immune system recognizes allogenous HLA molecules. Natural killer cells will become activated and kill all the transferred cells. Improvements could be made in this part of the therapy with the use of blockers of HLA's or using irreversible antagonists.

TCR-T cell therapy

Also in the specific use of TCR-T cell therapy many advantages can be made. Decrease in programmed cell death signaling will increase the life span of the transferred T cells, which would increase the efficacy of each injection with T cells. An example of these signals is programmed cell death 1 (PD-1). If this method appears to be successful, more death-inducing signals blocking antibodies could be used to increase the life span of T cells. PD-1 research suggests a reset of the immune interaction between tumor and host as the PD-1 therapy appears to restore the function of T cells with long term memory, which causes an ongoing anti-tumor effect even in the absence of treatment. A recurrence of tumors may reflect a disturbance in this equilibrium, where new administration will lead to a new balance and therefore tumor regression *(E.J. Lipson, S.L. Topalian, 2013)*.

Migration of the adoptive T-cells to the cancer site is crucial for the efficacy of therapy *(I. Marigo, L. Dolcetti, 2008)*. Therefore, new approaches could include co-expression of chemokines on the surface of CARs. The feasibility has been shown by *M.H. Kershaw, G. Wang et al in 2002.* The challenge is to target a specific chemokine secreted by the tumor and to design the appropriate receptor on the T cell. This would increase the efficacy of each transferred T cell.

Improvements in TCR-T cell therapy could be aimed at obtaining higher specificity to decrease collateral damage. When T cells are equipped with multiple receptors the specificity increases. Some of these receptors could be specific for tumor specific antigens, while other receptors induce inhibitory signals to 'endagered' tissue. When several tumor specific receptors can be placed on one T cell the tumor specificity would increase.

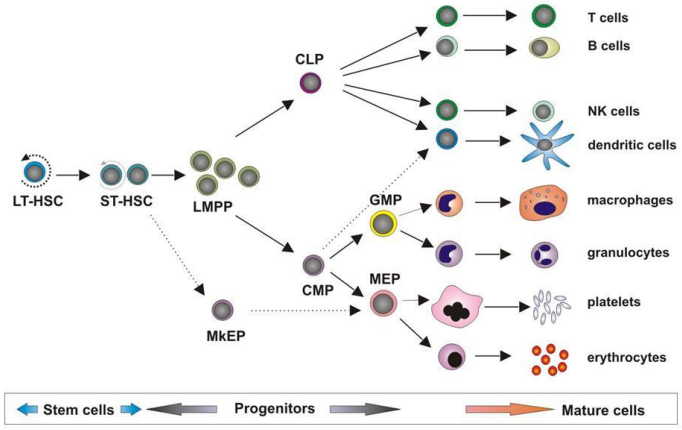
Not only increasing the specificity would improve the functioning of T cells, the effects of T cells measured in cytokine secretion and cytotoxitiy could be increased for optimal functioning. The conventional way of ensuring this is by optimizing the incubation circumstances. However, this could advance by means of including genes in the DNA that enhance cytotoxicity and cytokine secretion. Death inducing molecules can be targeted more specificly.

T cells transferred into the human body after an incubation time always have a decreased life span. This could be caused by the differentiation process of the cells. To increase the time in which the transferred T cells can be observed, less differentiated cells could be administered to acquire cells that will proliferate even after transfection. The concept of this idea will be discussed below.

The stem cell concept

Organism homeostasis requires a perfect balance between apoptosis and proliferation, between self-renewal and differentiation of stem cells. In the human body a hierarchy of cells is known, based on proliferative and differential capacities. The stem cell has the capacity of regenerating itself without losing its integrity and also has the capability of generating differentiated tissue as displayed in figure 5**.** It can divide in a daughter cell which differentiates and in a cell which can apply 'self-renewal.' So throughout the human body, cells in different stages can be found in every organ, discernible by surface molecules,

Fig. 5. *Capacity of the hametopoietic stem cell, differentiating into all types of cells from the hametopoietic system.*



anatomic location and function. Stem cells require very specific signaling to maintain their capacity for self renewal from a small environment called 'the stem cell niche.' Here the cell is nurtured and sensitive for signaling about when to proliferate. This is also the case in T cells where stem cells in bone marrow differentiate in all cells in the blood stream, including cells of the immune system (fig.?). Furthermore, T cells will differentiate in the thymus, hence the name, dependent on specific antigen presentation in the context of an MHC class II molecule, co-stimulatory signals and the strength of the TCR. The T cell can differentiate in a progressive continuum of sub-types, dependent on the signals received from the environment. Different models for T cell differentiation have been designed, however there is no evidence for which one of these is correct.

Identification of stem cell memory T cells

Differentiated T cells are continuously replaced by newly formed, less differentiated cells to renew the sytem and keep it vivid. Therefore it has been suggested that memory cells, that facilitate this renewal, contain stem cell like properties by being capable of renewal of T lymphocytes after antigen stimulation. Several characteristics have been found amongst memory cells, including self renewal and multipotency. The existing subset of T lymphocytes is described as a continuum and distinction is made by expression of different molecular markers. In the category of memory cells, there is a distinction between cells expressing CD62L and CCR7 positive and negative cells, central memory and effector memory cells respectively *(F. Sallusto, D. Lenig et al., 1999)*. The central memory subset is the one considered to posses stem cell like properties whereas the effector cells are thougth to undergo a limited number of divisions and becoming terminally differentiated.

Recently, an experiment on mice has discovered a population of T lymphocytes distinct from these central memory cells possessing more stem cell like properties. They have a similar phenotype with naïve T cells but also express antiapoptotic molecules and stem cell specific antigens. The identification of these cells in human remains to be accomplished.

The concept of stem cell T cells has lead to an interest in the idea of a T stem cell in the use of adoptive T cell therapy. For an effective treatment, less cells would be needed as the T cell can give rise to multiple T cells.

Attributes of stem cell memory T cells

The cells indentified in the above mentioned preclinical trial have some similarities with conventional memory cells but are distinct in multiple aspects. Here I will clarify the characteristics of stem cell memory T cells.

Similarities and differences between conventional memory T cells. After encountering an antigen, memory cells and the newly found memory stem cell T cells are transferring to T cells with an effector function in a relative short amount of time. That is to say, T_{SCM} have the memory capability similar to memory cells. Naïve cells take much longer and remain relatively quiescent. The response to

different types of cytokines differs amongst the subset of T cells. Memory T cells undergo proliferation in response to IL15 and IL7 *(M. Prlic, L. Lefrancois,2002),* unlike naïve T cells. T_{SCM} behave more like memory cells in that sense, as they undergo numerous divisions but still remain quite undifferentiated, as one would expect from a stem cell.

The identification of cell surface markers within one individual for 22 months shows that T_{SCM} is clearly a stably expressed cell type and not a transition from naïve to memory cell.

They represent the least differentiated cells in the subset of T cells. Comparing the transcriptome of the complete subset of T cells, a hierarchy of differentiation was defined. It pointed towards a linear model of T cell differentiation with at the basis naïve T cells with stem cells as the least differentiated memory cell.

Cytokine alternatives. As mentioned in the previous chapter, IL-2 has been used frequently to generate T cells ex vivo. However, late findings in the development of stem cell knowledge has shown that other molecules also have the potential of proliferating T cells, even without the robust pro-differentiating capacity that characterizes IL-2. The administration of IL-15 results in the generation of T cells similarly to naturally arising T_{CM} cells. Cells cultured in the presence of IL-15 have a greater antitumor response than cells cultured in IL-2. IL-21 is another molecule with the features to replace IL-2. IL-21 profoundly inhibits T cell differentiation and thereby stimulates the persistence of T_{SCM} like T cells. When cultured in IL-21, T cells have greater antitumor capacities compared to any other cytokine.

Enhanced self-renewal and multipotency. These qualities are defining for stem cells and therefore crucial for the argumentation of the existence of T_{SCM}. After investigating the different markers after stimulating with viral antigen, the T_{SCM} gave rise to both effector cells and memory cells, where some did proliferate but not differentiate.

Increased proliferation, survival and antitumor activity of T_{SCM}. T effector cells, currently used in adoptive T cell therapy, have poor proliferative capacity compared to T_{SCM}.

Application in ACT, reprogramming terminally differentiated cells to Tscm's

Adoptive t cell therapy increases in efficacy and has promising clinical results but in many patients the cells are incapable of showing a durable complete response. The reason for this could be terminal differentiation and exhaustion of T the cells *in vivo* as the number of detected T cells rapidly decreases as a function over time. As mentioned before, T cells that are transferred when being in a less differentiated state are correlated with an increased clinical response *(S.A. Rosenberg, M.E. Dudley, 2011).* ACT has been performed in mice using the less differentiated T_{SCM} and displayed enhanced survival and antitumor activity.

Since the discovery of induced pluripotent stem cells by Yamanaka et al. the path is paved for stimulation of certain embryonic markers to reverse differentiation of T cells and stimulate again to highly specific and effective antitumor T cells *(K. Takahashi, S. Yamanaki, 2006).* From the moment

this discovery was published, the implications are ongoing. Before this discovery, nuclei were transfected into oocytes to reprogram somatic cells *(I. Wilmut, A.E. Schieke, 1997).*It was known that there must be some factors involved in this reprogramming. Now it is clear that only 4 factors are required to induce pluripotent stem cells.

Conclusion

ACT has been performed in mice using T_{SCM} and displayed enhanced survival and antitumor activity. Combining the abovementioned discoveries, the use of induced pluripotent stem cells could increase the efficacy of ACT. It would overcome the problem of operating severely weakened patients to acquire tumor-infiltrating lymphocytes. This could imply that the specificity could be designed and is applicable in multiple patients as it is not bound to HLA molecules anymore.

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